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A2E, a byproduct of the visual cycle

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Abstract

A substantial portion of the lipofuscin that accumulates with age and in some retinal disorders in retinal pigment epithelial (RPE) cells, forms as a consequence of light-related vitamin A recycling. Major constituents of RPE lipofuscin are the di-retinal conjugate A2E and its photoisomers. That the accretion of A2E has consequences for the cell, with the adverse effects of A2E being attributable to its amphiphilic structure and its photoreactivity, is consistent with evidence of an association between atrophic age-related macular degeneration (AMD) and excessive lipofuscin accumulation.
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1. Introduction

Circumstantial evidence gathered over a number of years has implicated retinal pigment epithelial (RPE) lipofuscin in the etiology of atrophic age-related macular degeneration (AMD) (Delori, 1995; Delori, Goger, & Dorey, 2001; Dorey, Wu, Ebenstein, Garsd, & Weiter, 1989; Feeney-Burns, Hilderbrand, & Eldridge, 1984; Holz, Bellman, Staudt, Schutt, & Volcker, 2001; Holz, Bellmann, et al., 1999; von Rückmann, Fitzke, & Bird, 1997). Although the lipofuscin present in many non-dividing cell-types is thought to be derived in large part from lipid peroxidation products (Eldred & Katz, 1989; Eldred, Miller, Stark, & Feeney-Burns, 1982), more than 90% of the lipofuscin amassed in RPE cells originates from conjugates formed by visual cycle retinoids in photoreceptor cells (Katz, Drea, & Robison, 1986; Katz, Eldred, & Robison, 1987; Katz & Redmond, 2001). This material is then deposited in RPE cells subsequent to outer segment disc phagocytosis (Katz, Drea, Eldred, Hess, & Robison, 1986) and since it cannot be enzymatically degraded, it accumulates. RPE lipofuscin fluorophores are amassed with age (Delori et al., 2001; Feeney-Burns et al., 1984; Okubo et al., 1999; von Rückmann et al., 1997; Weiter, Delori, Wing, & Fitch, 1986; Wing, Blanchard, & Weiter, 1978) and the greatest accretion is in RPE cells underlying the macula (Dorey et al., 1989; Feeney-Burns et al., 1984; Weiter et al., 1986; Wing et al., 1978). The lipofuscin in RPE cells is also the source of the intrinsic fluorescence that emanates from the fundus (Delori, 1995; Delori, Dorey, et al., 1995). Indeed, monitoring of fundus autofluorescence by confocal ophthalmoscopy has established that zones of RPE exhibiting intense autofluorescence are prone to atrophy (Holz et al., 2001; Holz, Bellmann, et al., 1999; von Rückmann et al., 1997).

2. RPE lipofuscin fluorophores generated from visual cycle flux of all-*trans*-retinal

The only fluorophores of RPE lipofuscin that have been characterized as yet, are A2E (Ben-Shabat, Parish, et al., 2002; Eldred & Lasky, 1993; Parish, Hashimoto, Nakanishi, Dillon, & Sparrow, 1998; Ren, Sakai, & Nakanishi, 1997; Sakai, Decatur, Nakanishi, & Eldred, 1996), iso-A2E (Parish et al., 1998), and other less abundant photoisomers of A2E (Ben-Shabat, Parish, et al., 2002) (Fig. 1), all of which are generated by phosphate hydrolysis of the precursor, A2-PE, a

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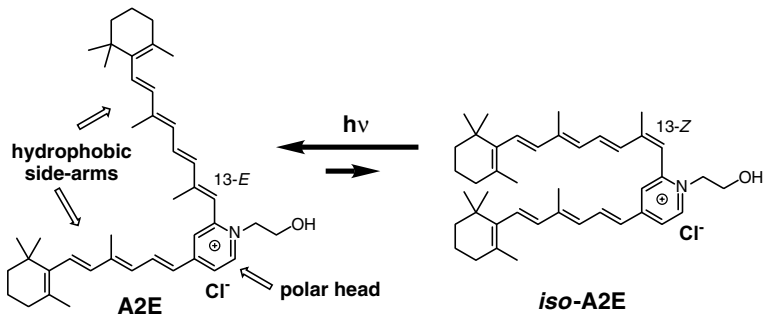

Fig. 1. Structure of the pyridinium bisretinoid, A2E. The hydrophobic side-arms derived from all-*trans*-retinal, together with a hydrophilic portion, confer amphiphilic properties on A2E. In vivo, the counterion of the pyridinium moiety is probably chloride. Iso-A2E, a *cis* olefin at the C13-14 position, is more streamlined than the wedge-shaped A2E. The photoisomers A2E and iso-A2E achieve equilibium at a ratio of 4:1.

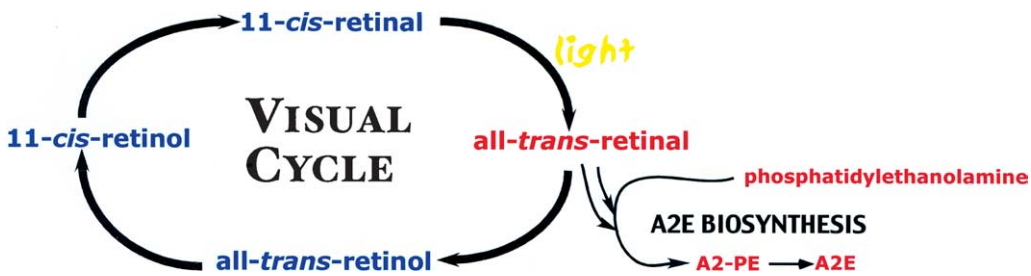

Fig. 2. The visual cycle and A2E formation. A2E biosynthesis begins in photoreceptor outer segments when all-*trans*-retinal leaves the visual cycle to react with phosphatidylethanolamine (ratio, 2:1). The precursor A2-PE is generated in a multi-step synthetic process, with A2E being released after phosphate hydrolysis.

phosphatidyl-pyridinium bisretinoid (Liu, Itagaki, Ben-Shabat, Nakanishi, & Sparrow, 2000) (Fig. 2). A2-PE forms as a byproduct of the visual cycle, its synthesis from all-*trans*-retinal occurring when molecules of all-*trans*-retinal, rather than undergoing reduction to retinol, react with phosphatidylethanolamine (PE) (Liu et al., 2000; Mata, Weng, & Travis, 2000; Parish et al., 1998). The initial Schiff-base conjugate (*N*-retinylidene-PE; NRPE) that forms between a single all-*trans*-retinal and PE (Liu et al., 2000; Sun, Molday, & Nathans, 1999; Weng et al., 1999) may serve as a substrate for ABCA4 (ABCR), the photoreceptor-specific ATP-binding cassette transporter (Molday & Molday, 1979; Molday, Rabin, & Molday, 2000; Papermaster, Schneider, Zorn, & Kraehenbuhl, 1978; Sun & Nathans, 1997; Sun & Nathan, 2001) that is thought to move all-*trans*-retinal to the cytosolic side of the disc membrane (Ahn, Wong, & Molday, 2000; Illing, Molday, & Molday, 1997; Sun & Nathans, 1997; Sun & Nathan, 2001; Weng et al., 1999) where it is accessible to retinol dehydrogenase, the enzyme responsible for its reduction to all-*trans*-retinol (Saari, Garwin, Van Hooser, & Palczewski, 1998). The reaction of NRPE with a second all-*trans*-retinal to form a molecule of A2-PE (Liu et al., 2000; Parish et al., 1998), is likely to occur very infrequently and probably only when circumstances, including high levels of illumination (Saari et al., 1998), increase the availability of all-*trans*-retinal. Accordingly, the observation that exposure to bright light favors A2-PE formation (Ben-Shabat, Parish, et al., 2002), is perhaps explained by light-driven release of all-*trans*-retinal and supports the concept of a relationship between cumulative light exposure and the accretion of A2E by RPE.

3. Evidence supporting a link between RPE lipofuscin and atropy

The relationship between lipofuscin accumulation and retinal degeneration is illustrated by Stargardt disease, a macular degeneration of juvenile onset that is caused by mutations in both alleles of the *ABCR* (*ABCA4*) gene (Allikmets et al., 1997; Shroyer, Lewis, Yatsenko, & Lupski, 2001; Shroyer, Lewis, Yatsenko, Wensel, & Lupski, 2001; Yatsenko, Shroyer, Lewis, & Lupski, 2001). Stargardt disease is also characterized by copious accumulation of RPE lipofuscin (Delori, Staurenghi, et al., 1995; Eagle, Lucier, Bernardino, & Yanoff, 1980; Lois, Holder, Fitzke, Plant, & Bird, 1999; Lopez, Maumenee, de la Cruz, & Green, 1990). Work with a mouse model of Stargardt disease, the *ABCR* null mutant, has shown that the abundant lipofuscin that accompanies a loss of ABCR activity is attributable, at least in part, to elevated A2E levels (Mata et al., 2001; Mata et al., 2000; Weng et al., 1999). Moreover, the means by which *ABCR* mutations predispose to RPE atrophy in Stargardt disease can be understood from what is known about the harmful effects of A2E on cell

function and viability. Several consequences of A2E accumulation have been described (Holz, Schutt, et al., 1999; Schutt, Davies, Kopitz, Holz, & Boulton, 2000; Sparrow, Nakanishi, & Parish, 2000; Sparrow, Parish, Hashimoto, & Nakanishi, 1999; Suter et al., 2000), all of which can probably be explained by two properties of the compound: its detergent-like structure and its photoreactivity.

4. A2E as an amphiphile

The hydrophobic side-arms of A2E, together with its positively charged hydrophilic head group, confer on A2E an amphiphilic structure (Sakai et al., 1996) (Fig. 1). There seems to be little doubt that the side-arms of A2E intercalate into the hydrophobic core of the membrane bilayer, since the emission maximum of cell-associated A2E corresponds most closely to that observed in non-polar solvents (Sparrow et al., 1999). Less clear is the behavior of the polar pyridinium moiety; the nature of its affinity for the counterion (probably chloride) is likely to be a factor influencing its movement with the lipid bilayer. For instance, the mode in which it penetrates the membrane may be influenced by electrostatic attraction with negatively charged phosphatidylserine (Raucher & Sheetz, 2001).

Studies using the plasma membrane as a model to evaluate the effects of A2E on a phospholipid bilayer, showed that when presented to membranes at high concentrations, A2E induced concentration dependent loss of membrane integrity, with membrane permeabilization being evidenced by the egress of lactate dehydrogenase from the cytoplasm and by the labeling of nuclei with a cell-impermeant dye (Sparrow et al., 1999). These observations were later confirmed in studies demonstrating the ability of A2E to solubilize the lipid bilayer of unilamellar vesicles (De & Sakmar, 2002). A2E-induced membrane blebbing provides further evidence of the tendency for A2E, at high concentration, to perturb membrane stability (Fig. 3). In these studies, a lentivirus vector was used to transduce wild-type GFP (Lai et al., 1999) in ARPE-19 cells such that the cytosol-filled membrane blisters were labeled as they formed. Work with other amphipiles has shown that the loss in cytoskeletal-membrane adhesion that accompanies bleb formation results from a physically induced biochemical change in the membrane (Dai & Sheetz, 1999). Whether A2E-mediated blebbing also represents a biochemical change in the membrane is not yet clear.

While unilamellar vesicles and the plasma membrane have been used experimentally as model lipid bilayers, interactions of A2E and the lysosomal membranes would presumably be more biologically relevant, since it

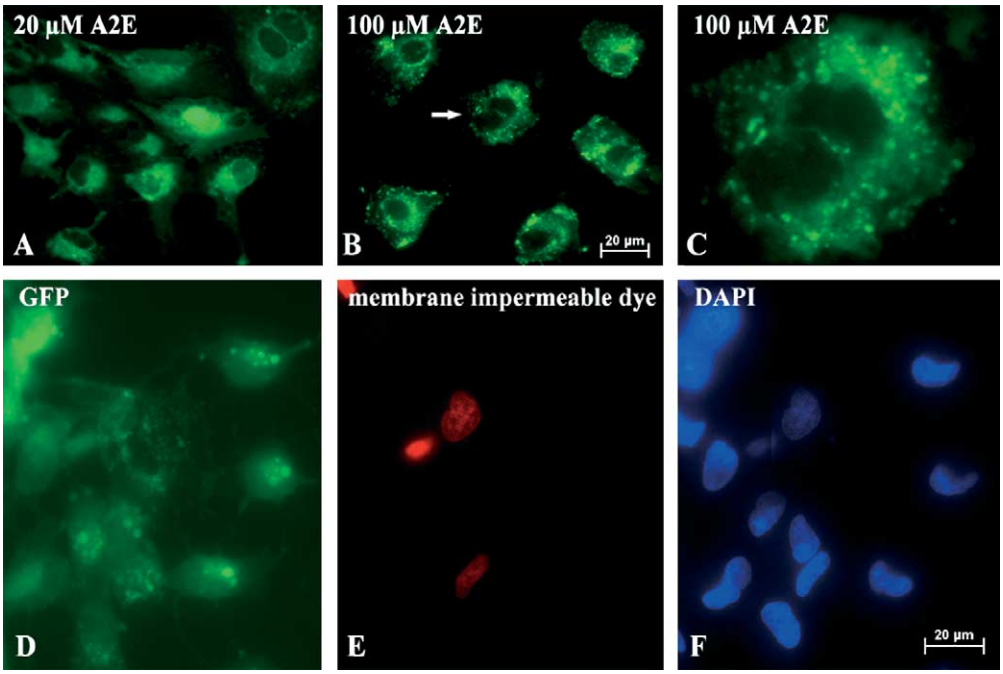

Fig. 3. A2E induces membrane blebbing. With the plasma membrane serving as a model bilayer, a lentiviral vector was used to transduce green fluorescent protein (GFP) in ARPE-19 cells; since wild-type GFP is localized to the cytoplasm and diffuses throughout, it served to fill the membrane blisters as they formed. A2E was introduced to cultured ARPE-19 cells at 20 μM (A) and 100 μM (B) concentrations. The small evaginations formed with 100 μM A2E are typical of membrane blebs. The cell at the arrow in (B) is shown at higher magnification in (C). At least during the early stages of bleb formation, the impermeability of the plasma membrane was retained since many cells exhibiting blebs (D), exclude a membrane impermeable dye (dead red; molecular probes) such that the dye does not label nuclei (E) that are otherwise stained by DAPI (4′,6-diamidino-2-phenylindole) (F).

is within intracellular lysosomal storage bodies that A2E accumulates. Accordingly, intracellular A2E reduces the acidity of these organelles (Holz, Schutt, et al., 1999), although it does not directly inhibit the activities of lysosomal enzymes (Berman, Schutt, Holz, & Kopitz, 2001). The latter observation is significant, since a failure of proteolytic digestion, brought about by the inhibitory effects of existing lipofuscin pigments, has been suggested to promote further lipofuscinogenesis. Interestingly, just as A2E accumulates in the lysosomal compartment in vivo, it becomes sequestered within the same organelles when introduced to cells in culture (Sparrow et al., 1999); but why it does so and why it remains there, is not clear.

5. Light-mediated damage to A2E-laden RPE

Light is not only essential to the formation of A2E, it may also be a factor involved in the adverse effects of A2E on RPE cells. Specifically, the blue region of the spectrum has a marked ability to induce apoptosis of A2E-laden RPE cells (Schutt et al., 2000; Sparrow & Cai, 2001; Sparrow et al., 2000) while RPE cells devoid of A2E remain unaffected. That green light is far less damaging, reflects a wavelength dependency consistent with the excitation spectrum of A2E. Investigations of the photochemical events that trigger the cell death have revealed that upon blue light irradiation, A2E self-generates singlet oxygen. The latter then proceeds to react with A2E at carbon–carbon double bonds along the retinoid-derived side-arms of the molecule to form epoxides (Ben-Shabat, Itagaki, et al., 2002; Sparrow et al., 2002) (Fig. 4). These highly reactive epoxides (A2E-epoxides), rather than singlet oxygen, may be the intermediates that ravage the cells, since singlet oxygen diffuses only a short distance in the cells (10–20 nm) yet DNA damage has been observed in blue light illuminated A2E-laden RPE cells (Sparrow, Zhou, & Cai, 2003). Moreover, much of the singlet oxygen generated by A2E is quenched by this bis-retinoid (Ben-Shabat, Itagaki, et al., 2002), and the resulting A2E-epoxides have been shown to induce DNA lesions, including oxidatively modified guanine bases (Sparrow et al., 2003). Nor is DNA the only cellular macromolecule with which A2E-epoxides are likely to form conjugates, because the latter are highly reactive electrophiles that are readily attacked by sulfhydryl and amino groups of proteins. In short, it is likely the generation of epoxide that enables A2E to be more phototoxic than would be expected on the basis of its photophysical properties. This is the case for some classes of potent photosensitizers that have relatively low singlet oxygen yields, but which react with singlet oxygen to generate harmful photoxidation products (Bunting, 1992; Delaey et al., 2000; Krieg & Bilitz, 1996). It is of interest that the natural antioxidants vitamins E and C, which have been reported to aid in the defense against progression of AMD (Age-Related Eye Disease Study, 2001; Beatty, Koh, Henson, & Boulton, 2000; Snodderly, 1995), were also shown to suppress A2E-epoxidation (Sparrow et al., 2003), suggesting that one mechanism by which these vitamins may protect is by restraining the damage that ensues from A2E photoxidation.

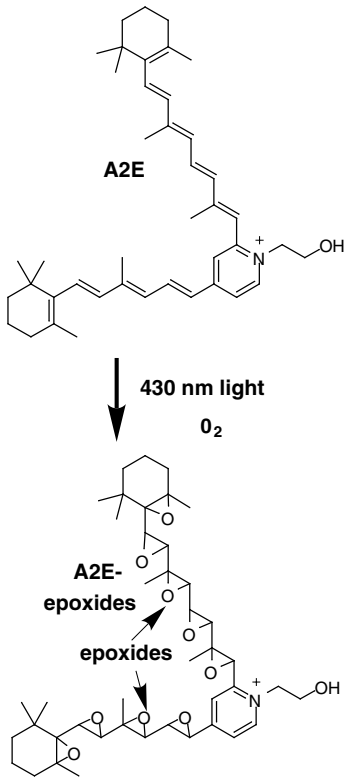

Fig. 4. A2E epoxidation. Irradiation of A2E leads to the generation of singlet oxygen with the latter adding to carbon–carbon double bonds to form epoxides (A2E-epoxides). Photoxidized A2E consists of a mixture of compounds exhibiting epoxides of varying numbers including the non-aoxirane illustrated here.

6. Additional putative fluorophores

Whether other fluorophores in addition to A2E and its photoisomers contribute to RPE lipofuscin is not yet clear. However, we have recently shown that all-*trans*-retinal at high concentration forms covalent bonds at three lysine residues in rhodopsin to generate fluorescent bis-retinoid adducts having emission spectra similar to A2E (Fishkin, Jang, Itagaki, Sparrow, & Nakanishi, 2003) (Fig. 5). While A2-PE formation is favored over this fluorescent all-*trans*-retinal-rhodopsin adduct (A2-rhodopsin), the generation of A2-PE appears to saturate at three equivalents (relative to rhodopsin) of retinal, with A2-rhodopsin being generated thereafter. The accumulation of A2-rhodopsin in seasoned photoreceptor outer segments may be significant in terms of reducing

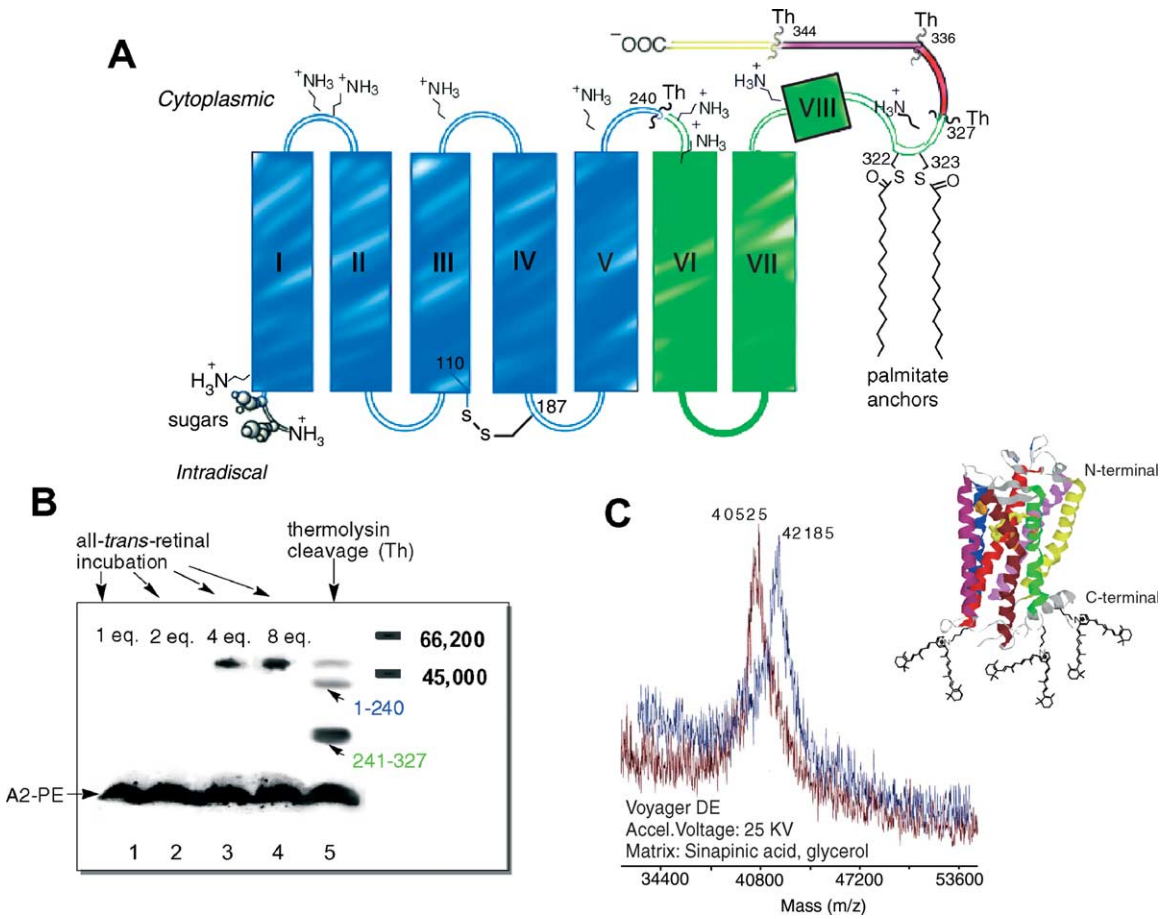

Fig. 5. Covalent adduct formation between all-*trans*-retinal and rhodopsin (A2-rhodopsin). (A) Structure of rhodopsin showing the eight helical segments together with the intradiscal and cytoplasmic loops. Sites at which thermolysin cleaves, are shown (Th). (B) Probing for A2-rhodopsin by denaturing SDS-PAGE with fluorescence detection (>525 emission). Isolated bovine outer segments were incubated with 1, 2, 4 or 8 equivalents of all-*trans*-retinal (relative to rhodopsin) (lanes 1–4). At 4 and 8 equivalents, fluorescent bands appeared that co-migrated with rhodopsin. Proteolysis by thermolysin (lane 5; sample as in lane 4), which is known to cleave rhodopsin at Ser-240 and Leu-328 (Imamoto, Kataoka, Tokunaga, & Palczewski, 2000), released the expected cleavage products corresponding to residues 1–240 (~26 kDa) and 241–327 (~12 kDa) of rhodopsin. The fragment comprising rhodopsin helices VI–VIII (241–327) exhibits a fluorescence intensity that is twice that of the I–V (1–240) fragment, suggesting that more all-*trans*-retinal adducts formed on the VI–VIII fragment. (C) MALDI-TOF mass spectral verification of covalent attachment of all-*trans*-retinal to rhodopsin. The 1660 Da shift (40,525 versus 42,185) relative to native rhodopsin is consistent with the addition of 6 retinals and removal of 6 water molecules and reveals the attachment of 3 bisretinoids (i.e. 2 retinal moieties at each of 3 positions) on rhodopsin. These all-*trans*-retinal adducts are likely to form by Schiff base reaction with the ε-amino group of lysine residues. Thus, given the more intense fluoresence of the 241–327 fragment (B), 2 bisretinoids must form at solvent exposed lysines (245, 248, 311 and 325) in the third cytoplasmic loop and/or the 8th helix, and 1 bisretinoid must be located in the 1–240 fragment.

the light sensitivity of rhodopsin; it may also, along with A2-PE, explain the necessity for constant replacement of photoreceptor outer segment membrane by the processes of disc assembly and shedding. Furthermore, the formation of these bisretinoid adducts in photoreceptor outer segments may be a sign that fluorescent peptide fragments bearing A2-moieties become deposited within RPE cells following outer segment phagocytosis.

7. Summary

Given the evidence that RPE lipofuscin is derived, for the most part, from retinoids that leave the visual cycle (Katz & Redmond, 2001), it is not surprising that the accumulation of RPE lipofuscin is most marked in central retina, the area having the greatest concentration of visual chromophore (Faulkner & Kemp, 1984; Liem, Keunen, & Van Norren, 1996; Tournow & Stilling, 1998), and thus the most pronounced capacity for photon catch. The foregoing, together with evidence indicating that life-time accumulations of lipofuscin can, under some circumstances, reach levels that provoke RPE atrophy, suggests that the light capturing function of the retina underlies mechanisms leading to macular degeneration. This concept is not new (Young, 1987; Young, 1988). Yet, studies aimed at forging a link between the incidence of AMD and life-time light exposure

have yielded inconsistent findings (Cruickshanks, Klein, & Klein, 1993; Darzins, Mitchell, & Heller, 1997; Taylor et al., 1992; West et al., 1989). Mounting evidence for the role of light in driving the formation of the RPE lipofuscin fluorophore A2E, together with the knowledge that this lipofuscin fluorophore can confer a susceptibility to blue light damage, begs a re-examination of this issue.

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